

EPOXIDE HYDROLASES: Mechanisms, Inhibitor Designs, and Biological Roles

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■ **Abstract** Organisms are exposed to epoxide-containing compounds from both exogenous and endogenous sources. In mammals, the hydration of these compounds by various epoxide hydrolases (EHs) can not only regulate their genotoxicity but also, for lipid-derived epoxides, their endogenous roles as chemical mediators. Recent findings suggest that the EHs as a family represent novel drug discovery targets for regulation of blood pressure, inflammation, cancer progression, and the onset of several other diseases. Knowledge of the EH mechanism provides a solid foundation for the rational design of inhibitors, and this review summarizes the current understanding of the catalytic mechanism of the EHs. Although the overall EH mechanism is now known, the molecular basis of substrate selectivity, possible allosteric regulation, and many fine details of the catalytic mechanism remain to be solved. Finally, recent development in the design of EH inhibitors and the EH biological role are discussed.

INTRODUCTION

Epoxide-containing compounds are ubiquitously found in the environment from both natural and man-made sources, and a large variety of aromatic and alkenic compounds are also metabolized to epoxides endogenously (1, 2). An epoxide (or oxirane) is a three-membered cyclic ether that has specific reactivity patterns owing to the highly polarized oxygen-carbon bonds in addition to a highly strained ring (3). Some reactive epoxides are responsible for electrophilic reactions with critical biological targets such as DNA and proteins, leading to mutagenic, toxic, and carcinogenic effects (4, 5). Although most epoxides are of intermediate reactivity, relatively stable at physiological pHs, and do not present acute dangers to cells, they still need to be transformed in a controlled manner (6). The catalytic addition of water to epoxides or arene oxides by epoxide hydrolases (EHs, E.C.3.3.2.3) to yield the corresponding 1,2-diols, or glycols (7), is only one of several ways that cells transform oxiranes. However, EHs are ubiquitous and hydration seems to be a common route of epoxide transformation. The reaction is energetically favorable, with water as the only cosubstrate.

The role of epoxide hydrolases seems to differ profoundly from organism to organism. Overall, these enzymes have three main functions: detoxification, catabolism, and regulation of signaling molecules. For microorganisms, EHs seem important in the catabolism of specific carbon sources from natural sources, such as tartaric acid or limonene (8, 9), as well as environmental contaminants such as epichlorohydrin (10). However, microbial EHs are mainly studied for their potential uses in chiral chemistry (11, 12). In plants, EHs have been characterized from several organisms (13–19), and the enzymes seem important in cuticle formation, responses to stresses, and pathogen defenses (15, 16, 20–22). EHs have also been characterized in several insects (23–27). Their roles in the catabolism of a key developmental chemical mediator, juvenile hormone (28), and in the detoxification of many plant chemical defenses have been studied (27, 29). In the vertebrate branch of the evolutionary tree, EHs have been mostly studied in mammals, which are emphasized in this review.

In mammals, there are several EHs, including cholesterol epoxide hydrolase (chEH), which hydrates the 5,6-oxide of cholesterol and other Δ^5 -epoxy steroids (30) and hepoxilin hydrolase (31). This review concentrates mostly on the soluble epoxide hydrolase (sEH) and microsomal epoxide hydrolase (mEH), which have been the most studied EHs over the past 30 years. These two enzymes were first distinguished by their subcellular localization, but they also have distinct and complementary substrate specificity (6, 32). Although these two enzymes are highly concentrated in the liver, they are found in nearly all tissues that were assayed for EH activity (6). These two enzymes are described to complement each other in detoxifying a wide array of mutagenic, toxic, and carcinogenic xenobiotic epoxides (6, 33); however, recent findings clearly implicate the sEH in the regulation of blood pressure and inflammation (34–40), and the mEH in xenobiotic metabolism and the onset of several diseases (41–45). Interestingly, inhibition of the sEH appears to be a potential therapeutic treatment for several diseases, including high blood pressure, atherosclerosis, and kidney failure (35, 38, 39, 46). A prerequisite for the development of potent inhibitors is an understanding of EHs mechanism of action. This mechanism has been studied since these enzymes were discovered more than 30 years ago; however, major breakthroughs were achieved in the past 10 years owing to the availability of recombinant EHs (47–49). Reviews have summarized the progress in unraveling EH mechanism several times during the past decade (6, 50–52). In this review, we outline our current understanding of EHs, catalytic mechanism. Furthermore, we focus on the development in the design of EH inhibitors and their use to understand the biological role of EHs in mammals.

MECHANISM

Formation of a Hydroxyl Alkyl-Enzyme Intermediate

The observation that both the mammalian mEH and sEH sequences are similar to a bacterial haloalkane dehalogenase and other related proteins was key in suggesting

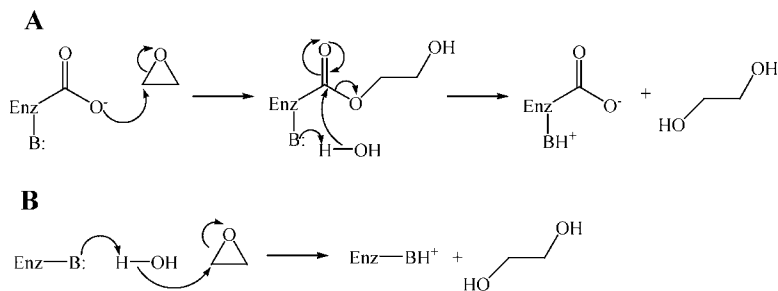


Figure 1 Proposed mechanism for epoxide hydrolase. (A) Two-step mechanism with the formation of a hydroxyl-alkyl-enzyme intermediate; (B) general-based-catalyzed direct hydration of the epoxide.

that both EHs have a similar mechanism to the bacterial enzyme (53) and that they are members of the α/β -hydrolase fold family of proteins (54). All the enzymes in this family are characterized by a nucleophile-histidine-acid catalytic triad and have a two-step mechanism involving the formation of a covalent intermediate (55, 56). This suggested that these EHs hydrolyze epoxides through the formation of a hydroxyl alkyl-enzyme intermediate as described in Figure 1A. Before this time, the generally accepted mechanism of EHs involved a general-based-catalyzed direct attack of water on the epoxide ring (Figure 1B; 57–59). Around the same time that the sequence analysis was done (54), Lacourciere & Armstrong (60) demonstrated the formation of a covalent intermediate for the mEH with a single turnover experiment (excess of enzyme) in H₂¹⁸O showing that the ¹⁸O was not incorporated in the formed glycol but rather in the protein. A second step was shown to incorporate the ¹⁸O in the product, even in H₂¹⁶O. Further evidence was gained through the isolation of the covalent intermediates for the sEH and mEH (61, 62). Chemical characterization of the enzyme-product intermediate indicated a structure consistent with an α -hydroxyl alkyl-enzyme (61).

The Catalytic Components

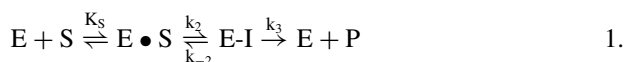
The amino acid residues forming the catalytic triad of the EHs were first identified from sequence alignment with the sequence of haloalkane dehalogenase (54, 63). Electrospray mass spectrometric analysis of the tryptic digestion of murine sEH incubated with substrate in H₂¹⁸O showed that the ¹⁸O was incorporated on a peptide containing Asp³³³. This confirmed the role of this residue as the nucleophile attacking the epoxide ring (64). Furthermore, the site-directed mutagenesis of this amino acid to a serine resulted in a total loss of activity, whereas its mutation to an asparagine yielded a mutant enzyme that reverted to the aspartate over time and therefore regained the activity (65). This observation was later measured for the mEH (66) and suggested the presence of a very basic water molecule near the catalytic site. In other α/β -hydrolase fold enzymes, the water molecule responsible

TABLE 1 Principal catalytic amino acids in human epoxide hydrolases.

| | Nucleophilic acid | Basic histidine | Orienting acid | Polarizing tyrosines |
|-----------|--------------------|--------------------|--------------------|---|
| Human mEH | Asp ²²⁶ | His ⁴³¹ | Glu ⁴⁰⁴ | Tyr ²⁹⁹ and Tyr ³⁷⁴ |
| Human sEH | Asp ³³⁴ | His ⁵²³ | Asp ⁴⁹⁵ | Tyr ³⁸² and Tyr ⁴⁶⁵ |

for the hydrolysis of the covalent intermediate is activated by a histidine/acid pair in a charge relay (55, 56). A histidine residue had been implicated in the catalytic mechanism of EHs years before (67). Site-directed mutation of histidine 431 for rat mEH and 523 for mouse sEH resulted in total loss of activity, indicating that these residues may function as a general base (58, 65). Furthermore, the rat mEH H431S mutant is still able to form the covalent intermediate but cannot hydrolyze it, showing that this histidine plays a role in activating the molecule of water (68). Based on the sequence alignments, the identification of the orientating acid residue of the catalytic triad was more speculative (54, 63, 69). The preparation of numerous site direct mutants of acid residues (66, 70, 71) has allowed the identification of Asp⁴⁹⁵ and Glu⁴⁰⁴ as the orientating acid for the rat sEH and mEH, respectively. Interestingly, for the rat mEH, the replacement of Glu⁴⁰⁴ with an aspartic acid results in a dramatically increased turnover rate (71). In the recent literature, numerous papers have reported the presence of similar catalytic triads (Asp/His/Asp or Glu) in other EHs from diverse origins by sequence alignments. We report in Table 1 the catalytic triad residues number of the human mEH and sEH active sites.

Early work indicated that both mEH and sEH catalyzed the *trans*-addition of water to epoxides through a general base catalysis (see 6 and references therein). Furthermore, the occurrence of a nucleophilic mechanism in the rate-determining step was strongly supported by the observed positive correlation between the rate of hydrolysis by mEH and the Hammett constant of substituted styrene oxides (57). Although these early findings agreed with the two-step mechanism described in Figure 1A, it raised the question of which step was rate limiting. Presteady-state kinetic analysis of epoxide hydration catalyzed by mEH (72) revealed that k_3 , the rate of hydrolysis of the hydroxyl alkyl-enzyme intermediate (E-I in Equation 1), was far slower than the rate of its formation (k_2).



Furthermore, it was found that the formation of the ester intermediate was reversible ($k_{-2} \neq 0$), indicating that the enzyme could stabilize the oxyanion in the alkylation reaction (first step). Other α/β -hydrolase fold enzymes have an "oxyanion hole" that stabilizes tetrahedral intermediates for the formation and hydrolysis of the covalent bond between the enzyme and the substrate (55, 56). The presence of such an oxyanion hole in sEH was proposed with a push-pull mechanism that

activated the epoxide by protonation or hydrogen bonding of the oxygen atom (69). Using chalcone oxides, which are substrates of sEH with a very low turnover rate (k_3 is very small), a slightly negative correlation between k_2 and the Hammett constant of *para*-substitutions was found, indicating that the formation of E-I was driven by a slight acidic mechanism (73). Furthermore, the low magnitude of the Hammett parameter suggested the presence of a weak charge that excluded the formation of a true carbonium ion or oxyanion, implying a general acido-basic-catalyzed process in the formation of E-I. The residues composing the oxyanion hole vary greatly inside the α/β -hydrolase fold family. For example, haloalkane dehalogenases have tryptophans (53), whereas esterases have two glycines (74). Over the years, based on other α/β -hydrolase fold enzymes, several residues were proposed and tested for being part of the oxyanion hole, but without success (58, 65, 66, 68, 70). The breakthrough was obtained with the acquisition of X-ray crystal structures of EHs (75–78). As shown in Figure 2 for the murine sEH, two tyrosine residues (381 and 465) located above the nucleophilic aspartate 333 (responsible for the formation of the E-I complex) are the best candidates for supplying general acid catalysis in the first half reaction (77). The mutation of either of these two tyrosines to phenylalanine results in a 90% decrease in activity (79). Furthermore, the kinetics of chalcone oxide hydrolysis show that both mutations decrease the binding (larger K_S) and the rate of formation of an intermediate (lower k_2), suggesting that both tyrosines affect epoxide polarization and facilitate ring opening (79). However, these two tyrosines are not implicated in the hydrolysis of the covalent intermediate (no change in k_3), suggesting that there is no intermediate to be stabilized in the hydrolysis step. This is consistent with the observation that the second half of the reaction is not reversible (60). Interestingly, these two tyrosines are conserved in EHs through evolution (78, 79), and the mutations of the equivalent residues in the mEH also resulted in dramatic loss of activity, like for the sEH (51, 79). The polarizing tyrosines of the human mEH and sEH are reported in Table 1.

The Catalytic Cycle

The above information is summarized in Figure 3. In the first step of the catalytic cycle of EHs, the epoxide quickly binds to the active site of the enzyme. Crystal structures show that the mouse sEH has a 25-Å-deep L-shaped hydrophobic tunnel, with the nucleophile aspartate located near the bend of the “L” and both ends open to the solvent (76). It is not known if the substrate enters the active site by crawling down the tunnel or if the cap of the active site opens to let the substrate in then closes for the catalysis. The latter possibility is supported by the fact that the fluorescence of mEH changed significantly upon substrate binding (60), suggesting a large movement in the enzyme structure. However, there are other possible explanations for the observed change. Examination of the crystal structure of the murine sEH with the inhibitor N-cyclohexyl-N'-decylurea bound (Figure 4) shows that there are hydrophobic pockets on either side of the central catalytic

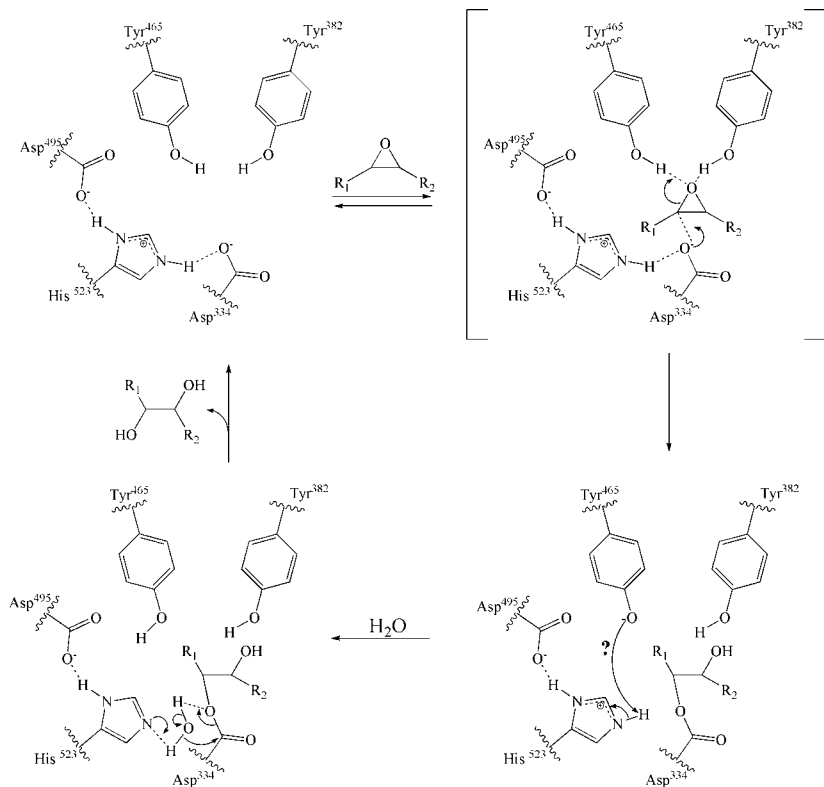


Figure 3 Catalytic mechanism of EH. The amino acid residue numbers correspond to the human sEH.

residues (Asp³³³ and His⁵²³). This suggests that Van der Waals interactions make a significant contribution to substrate binding (77). However, such an analysis also indicates a number of potential hydrogen bonding sites primarily located on the surface opposite of Asp³³³, which could be important in the formation of the intermediate (top right of Figure 3).

The substrate epoxide is polarized by two tyrosine residues (382 and 465), which hydrogen bond with the epoxide oxygen. At the same time, the nucleophilic carboxylic acid of Asp³³⁴, present on the side of the catalytic cavity opposite to the tyrosines, makes a backside attack on the epoxide, usually at the least sterically hindered and most reactive carbon. The nucleophilic acid is oriented and activated by His⁵²³, a second carboxylic amino acid (Asp⁴⁹⁵) and possibly other amino acids in the catalytic site for this attack. A recent study based on molecular dynamics simulations (80) suggests that the protonation of His⁵²³ is essential for the right orientation of Asp³³⁴; however, no experimental proofs exist yet. Because the mEH has a higher optimal pH for activity (pH 8.0–9.0) than the sEH (pH 7.0–7.5)

(32), the corresponding His in mEH is probably not protonated (80). The opening of the epoxide results in an ester bond between the enzyme carboxylic acid and one alcohol functionality of the diol. This is termed the hydroxyl alkyl-enzyme intermediate (bottom right of Figure 3). An important unanswered question is where the oxygen of the epoxide catches the hydrogen to form a hydroxyl because it was determined that an oxyanion is not formed (73). It was proposed that the proton came from one of the tyrosine side chains (77), as it is shown in Figure 3, and that the formed tyrosinate ion is stabilized by edge-to-face π -interactions with surrounding aromatic residues (79). However, the presence of a tyrosinate ion has yet to be proven. Furthermore, it could be argued that the high pKa (~ 10) of the tyrosine side chain makes it difficult for the phenolate to form at the pH (7.4 for sEH and 8.0–9.0 for mEH) at which the reaction is catalyzed (51). It would be intellectually satisfying if the hydrogen come from the protonated His⁵²³, especially because this histidine should be a base (not protonated) for the second half of the catalytic reaction (80). However, crystal structures show that this histidine residue is on the wrong side of the catalytic site to directly donate its proton to the epoxide (77). Therefore, a proton shuttle mechanism was proposed to transfer the proton from the histidine to the tyrosine (80), but this proton transfer pathway has yet to be shown.

Once the covalent hydroxyl alkyl-enzyme is formed, the histidine moves far enough from the nucleophilic acid (now ester) to allow a water molecule to be activated by the acid-histidine pair (bottom left of Figure 3). This movement may account for the fluorescence shift during the enzyme reaction (60). The activation of the water can occur only if the histidine is not protonated (80). This very basic water attacks the carbonyl of the ester, releasing the diol product and the original enzyme. As we described above, a variety of lines of evidence support this mechanism for both the mammalian microsomal and soluble EH and EHs from numerous other organisms. Interestingly, a few reports suggest that the cholesterol epoxide hydrolase has a different mechanism (see below).

The Cholesterol Epoxide Hydrolase

The chEH is the other EH located in the microsomal fraction (81, 82). Because this enzyme has not been purified to homogeneity or been cloned (33), little is known about it. Unlike mEH and sEH, which have a wide range of substrate specificities (6), chEH is very specific for cholesterol 5,6-oxide (82). The enzyme shows a fivefold preference for the α - over the β -diastereomer (83). The exact mechanism of catalysis of the chEH is not well known, but several lines of evidence suggest a mode of action different than the one described above for the sEH and mEH. First, its size is too small (84) to be a classical α/β -hydrolase fold enzyme (52, 55, 56). Furthermore, unlike mEH or sEH, chEH appears to hydrolyze cholesterol oxides via a positively charged transition state (85). Although covalent hydroxyl alkyl-enzyme were isolated from sEH and mEH (see above), Müller and collaborators were unable to isolate any covalent intermediate for chEH (62). All these results suggest that chEH hydrolyzes its substrate through a one-step general base

mechanism similar to the one described in Figure 1B. This hypothesis is supported by the recent report of the structure for the limonene-1,2-epoxide hydrolase from *Rhodococcus erythropolis* that has a one-step general-based-catalyzed direct hydration of the epoxide (86).

Quaternary Structures

Analysis of the primary structure suggests that the sEH gene (EPXH2) was produced by the fusion of two primordial dehalogenase genes; the C-terminal sEH domain has high homology to haloalkane dehalogenase, whereas the N-terminal domain is similar to haloacid dehalogenase (HAD) (54, 69). This gene fusion hypothesis was recently supported by an X-ray crystal structure of the mouse and human sEH that exhibit a domain-swapped architecture (Figure 5) where both domains of each monomer are separated by a proline-rich linker (76, 87). Furthermore, the three-dimensional (3-D) structures confirmed that unlike the mEH, the sEH is a homodimer with with a monomeric unit of 62.5 kDa as determined from biochemical analysis (6, 32). The C-terminal domain of one subunit interacts with both the C- and N-terminal domains of the other monomer. Beside the physical interaction between the two C-terminal domains, which contain the EH activity, no cooperative allosteric effects have been reported for the sEH activity (6). The fact that the C-terminal catalytic cavities are not close to any interdomain or interprotein interface may explain the lack of cooperativity in epoxide hydrolysis (76). Alternatively, it was found that in solution the monomer and dimer sEH are active (88, 89), suggesting a natural equilibrium between the two forms of sEH. Although a dissociation constant for the dimer formation has yet to be measured, it is possible that, under the conditions where sEH activity is generally measured (low nanomolar), the enzyme could be mainly in its monomeric form, thus preventing the detection of any possible allosteric effects.

Analysis of the sEH crystal structure reveals that while the C-terminal domain containing the EH activity adopts an α/β -hydrolase fold as expected, the N-terminal domain adopts an α/β -fold similar to HAD with a 15-Å-deep catalytic cavity with catalytic residues properly oriented for catalysis (76, 87). However, no dehalogenase activity was detected, and the N-terminal domain was first thought to only stabilize the formation of the dimer, and thus qualified as a vestigial domain (76). This hypothesis was supported by the fact that sEH orthologues in plants lack the N-terminal domain and are monomeric (69). However, the amino-terminal catalytic DXDX(T/V) motif of HAD has been used to describe an enzyme class that includes numerous phosphatases (90, 91), suggesting a possible catalytic activity for the N-terminal domain. Recently, a magnesium-dependent hydrolysis of a phosphate ester was associated with this domain of sEH (92, 93). The human sEH crystal structure supports a mechanism for this phosphatase activity similar to the one previously described for phosphatases of the HAD family (87). Interestingly, the sEH is found to hydrolyze the monophosphates of dihydroxy stearic acid yielding 1,2-diols similar to those obtained from the hydrolysis of stearic acid

epoxides by the sEH (93). Although sEH inhibitors do not influence the phosphatase activity, kinetic analysis revealed a positive cooperative Hill coefficient of ~ 2 for the hydrolysis of the monophosphate of dihydroxy stearic acid, suggesting an allosteric interaction between the two monomers (93). The recent 3-D structure of the human sEH reveals a ~ 14 -Å-long hydrophobic tunnel sufficiently large to accommodate the binding of an aliphatic substrate with one end at the active site and the other end near the interface of the N- and C-terminal domains, supporting the observed positive cooperative kinetics (87).

The mEH (EXPH1) does not have a large N-terminal domain similar to the sEH, but instead possesses a strongly hydrophobic transmembrane domain of approximately 20 residues, which anchors the protein to cellular membranes (94, 95). Although mEH activity is not completely lost after the removal of this anchor, the resulting protein is not soluble (95), suggesting a strong association of mEH to the membrane. The mEH is found to be tightly associated with phospholipids (96, 97), suggesting a complex binding between mEH and cellular membranes. At this time, little is known beyond these findings about how this enzyme is bound to the membrane. In liver, mEH is found to reside on both the smooth endoplasmic reticulum (ER; 98) and the plasma membrane (99, 100). Complicating matters, the topological orientation of mEH in the membranes appears to be different in the ER (catalytic C-terminal domain facing the cytosol) and in the plasma membrane (C-terminal facing the extracellular medium) (101, 102). A recent study using recombinant enzymes showed that mEH could associate nonspecifically with several P450s, resulting in an activation of the mEH activity; however, it is not known at the molecular level how this association occurs (103). Finally, the mEH was reported to be a subunit of two multiprotein complexes: a Na^+ -dependent bile acid transport (99, 104) and an antiestrogen binding site (105); however, nothing is yet known about how mEH interacts within these complexes and how it regulates their activities.

INHIBITOR DESIGNS

Specific enzyme inhibitors are important research tools to help understand the catalytic mechanism of an enzyme and the pathologies that may be associated with dysfunctions of this enzyme. This statement has been particularly true over the past few years for sEH, and the recent design of potent inhibitors for sEH has opened the door to new therapies for hypertension and inflammation (34–40). To start with a historic point of view, the first inhibitors discovered for the sEH and mEH were epoxide-containing compounds (Figure 6) (see 6 and references therein). However, most of these compounds are in fact substrates of the corresponding EH with a relatively low turnover that gives only a transient inhibition *in vitro* and are inefficient in cell cultures and *in vivo* (6, 73, 106, 107). A widely used mEH inhibitor, trichloropropene oxide, not only reacts with many proteins directly but

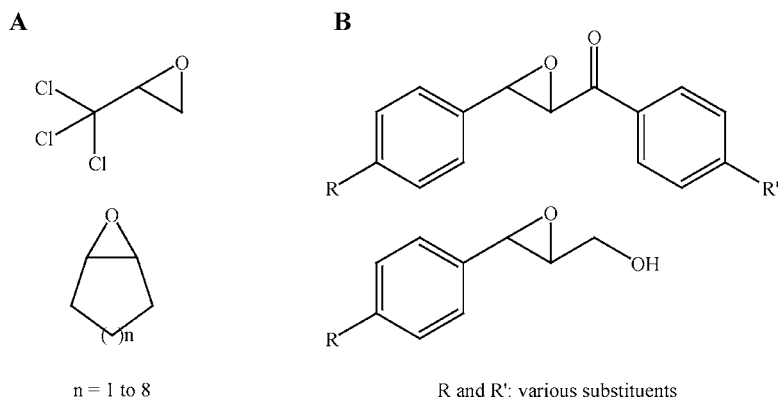


Figure 6 Epoxide-containing inhibitors of (A) the mEH and (B) the sEH.

it is rapidly turned over by the mEH (108). The mEH and sEH activities are found to be inhibited by Hg^{2+} and Zn^{2+} , and the sEH is also inhibited by Cu^{2+} and Cd^{2+} (109). The inhibition of the human mEH by Zn^{2+} is found to be competitive with a K_i of $\sim 60 \mu\text{M}$, whereas the inhibition of the human sEH is noncompetitive with a K_i of $\sim 20 \mu\text{M}$ (109). This divalent cation is found to also inhibit the phosphatase activity of the sEH (93). One could hypothesize that the binding of Zn^{2+} at the Mg^{2+} site in the N-terminal domain resulted in loss of both activities through some as yet unknown allosteric mechanism that is suggested by the sEH quaternary structure (see above). During inflammation, the concentrations of divalent cation metals, especially zinc, increase in the liver (110), suggesting that the binding of Zn^{2+} could be a simple way for the organism to naturally reduce the sEH activity that was shown to be proinflammatory (34, 38).

Approximately a decade ago, valpromide treatment was reported to affect the normal metabolism of carbamazepine by inhibiting the mEH *in vivo* (111, 112). The anticonvulsant properties of this compound could cause undesirable secondary effects in experimental systems if used as mEH inhibitor, but other unsubstituted amides could be used (113). Recently, primary ureas, amides, and amines were described as mEH inhibitors (Figure 7A; 114). The most potent inhibitor obtained, elaidamide, has a mix of competitive and noncompetitive inhibition kinetics with a K_i of 70 nM. It is efficient *in vitro* (114); however, its fast turn over by amidases limits its use *in vivo*, underlying the need of new potent and stable mEH inhibitors.

1,3-Disubstituted ureas, carbamates, and amides (Figure 7B) were recently described as new potent and stable inhibitors of sEH (115). These compounds are competitive tight-binding inhibitors with nanomolar K_i that interact stoichiometrically with purified recombinant sEH (115, 116). Crystal structures show that the urea inhibitors establish hydrogen bonds and salt bridges between the

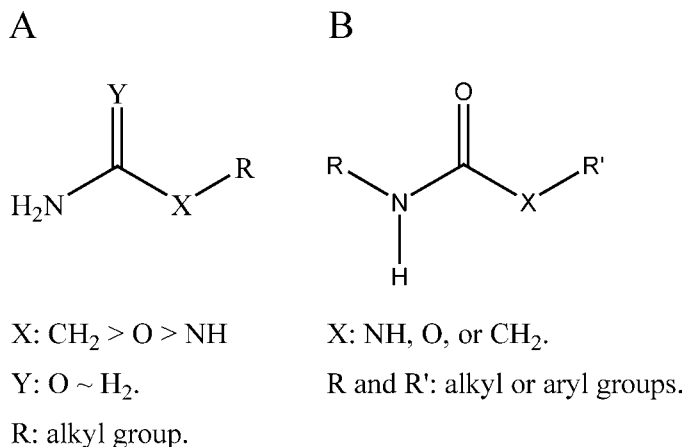


Figure 7 Structure of nonoxide-containing inhibitors of (A) the mEH and (B) the sEH.

urea functionality of the inhibitor and residues of the sEH active site, mimicking the intermediate formed during the enzymatic epoxide ring opening, as described in Figure 3 (76, 77, 87). Furthermore, the side chains of the inhibitors (R and R') need to be hydrophobic to bind tightly in the hydrophobic catalytic site, as shown in Figure 4 (76, 77). Interestingly, because of the presence of a methionine residue (Met³³⁷) pointing into the catalytic cavity, the orientation of the urea inhibitors is reversed in the human sEH compared with the mouse enzyme (87). Using classical quantitative structure activity relationship (QSAR), 3-D-QSAR, and medicinal chemistry approaches, the structure of these inhibitors were improved to yield compounds that have an order of magnitude better inhibition potency (116–119). This new generation of sEH inhibitors display on one side of the urea functionality secondary and tertiary pharmacophores at 5 and 11 atoms away from the urea carbonyl group, respectively (119). These inhibitors efficiently inhibit epoxide hydrolysis in several in vitro and in vivo models (38–40, 115, 120). The beneficial biological effects observed are discussed below.

BIOLOGICAL ROLES

The biological role of any enzyme is intimately linked to the substrates the enzyme transforms. Substrate specificity is probably the best way to distinguish between the mEH and sEH. These two enzymes have been found to hydrolyze a broad and complementary range of substrates (6, 32). In general, mEH seems to prefer mono- and *cis*-disubstituted epoxides, whereas the sEH prefers *gem*-di-, *trans*-di-,

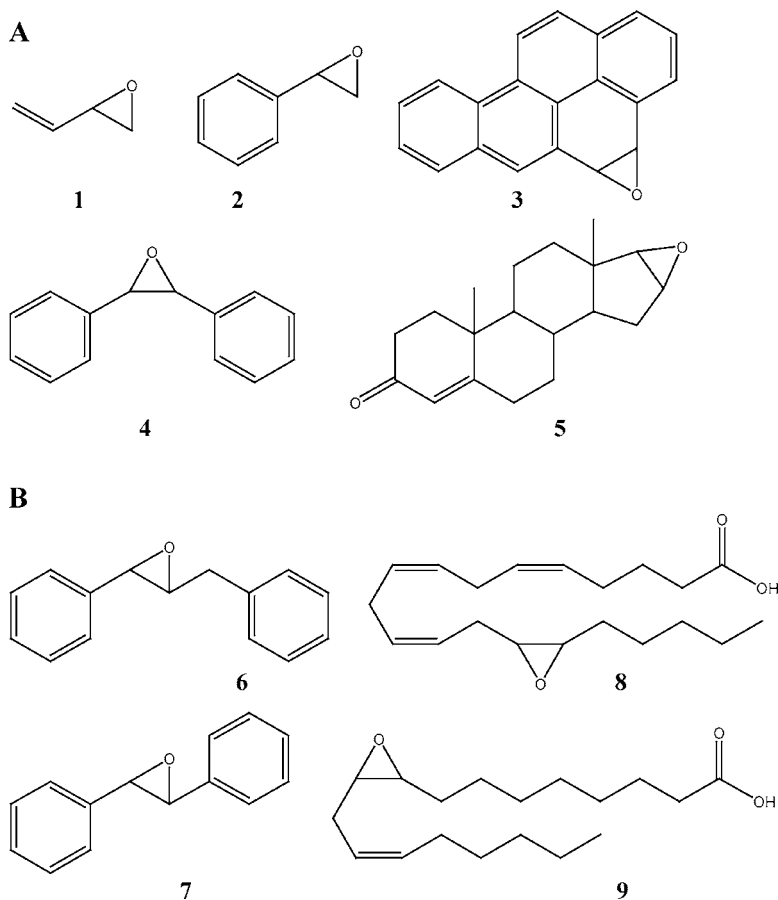


Figure 8 Structure of typical substrates hydrolyzed by (A) the mEH and (B) the sEH.

cis-di-, tri-, and tetra substituted epoxides (32). The latter enzyme hydrates epoxide on cyclic system very poorly (107). A few examples of mEH and sEH substrates are shown in Figure 8.

mEH is a key hepatic enzyme involved in the metabolism of numerous xenobiotics, such as 1,3-butadiene oxide **1**, styrene oxide **2**, and benzo(α)pyrene 4,5-oxide **3** (6, 33, 52). In addition, mEH is likely involved in the extrahepatic metabolism of these agents (33, 121, 122). Styrene **2** and *cis*-stilbene **4** oxides are widely used as surrogate substrates for mEH (32). The mEH action is part of a detoxification process for most of the substrates (6, 33). This detoxification action is illustrated by the example of a man who had a defect in mEH expression and suffered from acute and severe phenytoin toxicity (123). However, in some cases, such as for

benzo(α)pyrene 4,5-oxide **5**, diol formation can lead to the stabilization of a secondary epoxide, increasing the mutagenic and carcinogenic potential of the product (124). The procarcinogenic role of mEH was illustrated in mEH knockout mice that were less sensitive to the carcinogenic activity of 7,12-dimethylbenz[α]anthracene than control mice (125). Furthermore, in human populations, polymorphism in the mEH gene is associated with the onset of numerous cancers (42–44, 126, 127). The role of mEH in xenobiotic metabolism is probably also linked to the observed relationship between mEH polymorphism and emphysema (41) or Crohn's disease (45).

Despite the fact that mEH knockout mice do not present an obvious phenotype (125), there are several new lines of evidence suggesting an endogenous role for this enzyme. A potential role of mEH in sexual development is supported by the fact that androstene oxide **5** is a very good mEH substrate (128), and that mEH is an apparent subunit of the antiestrogen-binding-site (105). Such a role could be related to the observed relation between mEH polymorphism and spontaneous abortion (129) or preeclampsia (130). Furthermore, mEH is well expressed in ovaries (131), especially in follicle cells (132). During the past decade, mEH was also described as mediating the transport of bile acid in the liver (133, 134). The mechanism by which mEH participates in this transport is not known. Potent mEH inhibitors could provide new tools to better understand the multiple roles of this enzyme.

sEH was thought to participate in the metabolism of xenobiotics, like the mEH; however, there is no evidence supporting this hypothesis in vivo in mammals (6, 52). Radioactive aromatic epoxides, such as *trans*-diphenyl-propene oxide **6** and *trans*-stilbene oxide **7**, are classically used as surrogate substrates for this enzyme in vitro (32, 135). On the other hand, the sEH is clearly involved in the metabolism of arachidonic epoxides (**8**, also called epoxyeicosatrienoic acids or EETs) and linoleic acid epoxides (**9**, also called leukotoxins) both in vitro and in vivo (34, 35, 136, 137). The sEH hydrates all of these epoxy-fatty acids with high V_M and low K_M . The sEH-dependent transformation of EETs decline as the epoxide approaches the carboxyl terminal (i.e., 14,15-EET is hydrolyzed \sim 20-fold faster than 8,9-EET and 5,6-EET is hydrolyzed very slowly), whereas both mono-epoxides of linoleic acid are hydrolyzed at similar rates (138, 139). Although epoxy-fatty acids are relatively poor substrates for mEH compared to sEH (138), the former enzyme hydrolyzes them with a high enantioselectivity, whereas the latter shows little or no enantiomeric preference (140, 141).

The EETs, which are endogenous chemical mediators (142), act at the vascular, renal, and cardiac levels to regulate blood pressure (143, 144). The vasodilatory properties of EETs are associated with an increased open-state probability of calcium-activated potassium channels, which lead to hyperpolarization of the vascular smooth muscle (145). Hydrolysis of the epoxides by sEH diminishes this activity (146). The sEH-dependent hydrolysis of EETs also regulates their incorporation into coronary endothelial phospholipids, suggesting a regulation of endothelial function by sEH (147). Recently, blood pressure reduction was achieved

in the spontaneous hypertensive rat (SHR) and in angiotensin II-induced hypertension rat models with pharmacological sEH inhibition (35, 39). Additionally, male knockout sEH mice have significantly lower blood pressure than wild-type mice (36), further supporting the role of sEH in blood pressure regulation and sEH inhibition as a potential new therapeutic treatment for hypertension.

The EETs also display antiinflammatory properties in endothelial cells (37, 148). In contrast, diols derived from epoxy-linoleate (leukotoxin) perturb membrane permeability and calcium homeostasis (34), which results in inflammation that is modulated by nitric oxide synthase and endothelin-1 (149, 150). Micromolar concentrations of leukotoxin reported in association with inflammation and hypoxia (151) depress mitochondrial respiration in vitro (152) and cause mammalian cardio-pulmonary toxicity in vivo (150, 153, 154). Leukotoxin toxicity presents symptoms suggestive of multiple organ failure and acute respiratory distress syndrome (ARDS) (151). In both cellular and whole organism models, leukotoxin-mediated toxicity is dependent on epoxide hydrolysis (34, 115), suggesting a role for sEH in the regulation of inflammation. Treatment with sEH inhibitors increases EET levels in cell cultures and reduces indicators of vascular inflammation (38, 155), suggesting that sEH is a potential therapeutic target for the treatment of several vascular inflammatory diseases, including atherosclerosis and kidney failure (38, 46). Inhibition of the sEH seems to result in general antiinflammatory properties in many systems.

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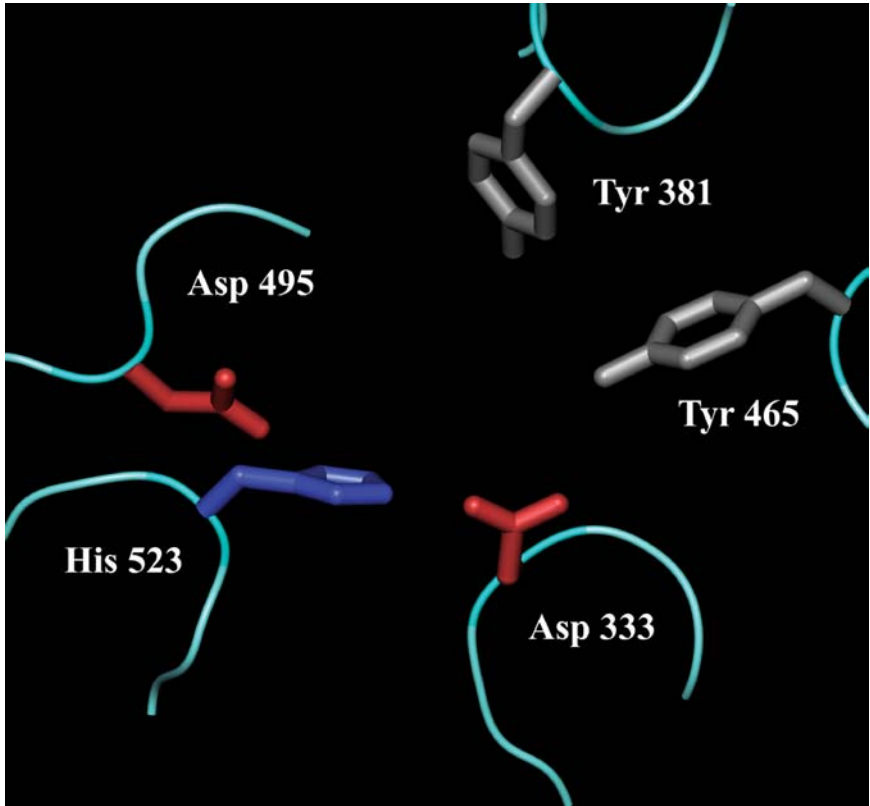


Figure 2 Structure of the active site of the mouse sEH showing the presence of two tyrosine residues, 381 and 465 (*gray*), positioned opposite of the catalytic triad (Asp³³³ in *red*, His⁵²³ in *blue*, and Asp⁴⁹⁵ in *red*). Structure obtained from Reference 76.

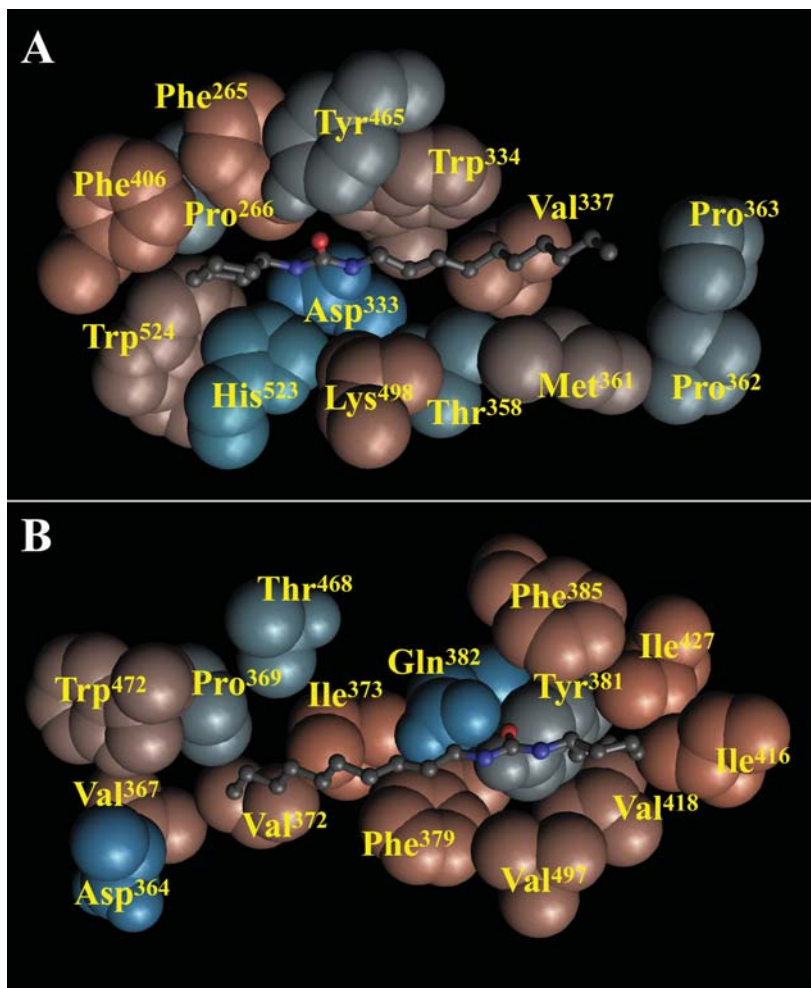


Figure 4 Hydrophobicity map of the mouse sEH substrate binding pocket cocrystallized with the inhibitor 1-cyclohexyl-3-dodecyl urea (77). Amino acid side chains within 6 Å of the inhibitor are displayed as space-filling models. The residues shown in bright red and blue are the urea oxygen and nitrogens, respectively. A color gradient of brown to blue indicates degrees of hydrophobicity. Panel A shows a view of the catalytic pocket from the inside of the enzyme toward the outside, and panel B shows the opposite view. A series of hydrophilic residues are observed on the “top” side of the channel (Phe²⁶⁵, Pro²⁶⁶, Trp³³⁴, Val³³⁷, Pro³⁶³, Pro³⁶⁹, Ile³⁷³, Phe³⁸⁵, Phe⁴⁰⁶, Ile⁴²⁷, Thr⁴⁶⁸, Trp⁴⁷²), whereas the “bottom” of the channel is very hydrophobic (Thr³⁵⁹, Met³⁶¹, Pro³⁶³, Val³⁷², Phe³⁷⁹, Ile⁴¹⁶, Val⁴¹⁸, Val⁴⁹⁷, Lys⁴⁹⁸, Trp⁵²⁴), with the exception of the catalytic aspartate and histidine (Asp³³³ and His⁵²³). This structural analysis indicates that a number of potential hydrogen bonding sites (Tyr³⁸¹, Gln³⁸², Tyr⁴⁶⁵) are observed in the substrate binding pocket of the soluble epoxide hydrolase, primarily located on the surface opposite Asp³³³.

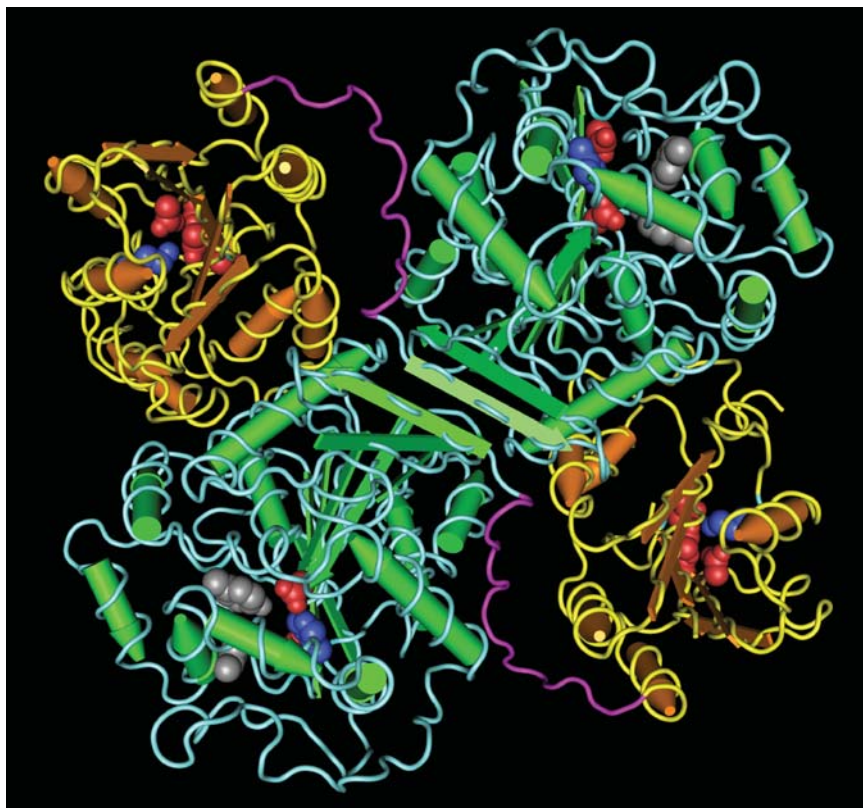


Figure 5 Structure of the mouse sEH dimer (76). The N-terminal domains (residues Arg⁴-Gly²¹⁸) are in yellow-orange, the C-terminal domains (residues Val²³⁵-Ala⁵⁴⁴) are in blue-green, and the proline-rich linker (Thr²¹⁹-Asp²³⁴) is in magenta. Catalytic residues for both the C- and N-terminal domains are displayed as space-filling residues with blue for positive charge, red for negative charge, and gray for neutral. The alternating helices and the beta sheet “floor” typical of the α/β -hydrolase fold enzymes is clearly shown in the C-terminal domain.

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